

## CLAIMS

What is claimed is:

1. A method for determining an unknown starting quantity of a target nucleic acid sequence in a test sample, the method comprising the steps of:
  - a) amplifying the unknown starting quantity of the target nucleic acid sequence in the test sample;
  - b) amplifying a plurality of known starting quantities of a calibration nucleic acid sequence in respective calibration samples;
  - c) determining a respective threshold value for each of the known starting quantities of the calibration nucleic acid sequence in the calibration samples and for the target nucleic acid sequence in the test sample, wherein the threshold value is determined for each nucleic acid sequence in a respective sample by:
    - i) measuring, at a plurality of different times during amplification, at least one signal whose intensity is related to the quantity of the nucleic acid sequence being amplified in the sample;
    - ii) deriving a growth curve from the measurements of the signal;
    - iii) calculating a derivative of the growth curve;
    - iv) identifying a characteristic of the derivative;

v) determining a threshold value associated with the characteristic of the derivative;

5 d) deriving a calibration curve from the threshold values determined for the known starting quantities of the nucleic acid sequence in the calibration samples; and

10 e) determining the starting quantity of the target nucleic acid sequence in the test sample using the calibration curve and the threshold value determined for the target sequence.

2. The method of claim 1, wherein each of the threshold values comprises a cycle number.

15 3. The method of claim 1, wherein each of the threshold values comprises an elapsed time of amplification.

20 4. The method of claim 1, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a positive peak of the second derivative.

25 5. The method of claim 1, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the

characteristic comprises a negative peak of the second derivative.

5 6. The method of claim 1, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a zero crossing of the second derivative.

10 7. The method of claim 1, wherein the step of calculating a derivative of the growth curve comprises calculating a first derivative of the growth curve, and wherein the characteristic comprises a positive peak of the first derivative.

15 8. The method of claim 1, wherein the step of calculating a derivative of the growth curve comprises calculating second derivative values of the growth curve at a number of different measurement points in the reaction to yield  
20 a plurality of second derivative data points, the characteristic comprises a positive peak of the second derivative, and the step of determining the threshold value associated with the positive peak comprises:

- 25 i) fitting a second order curve to the second derivative data points; and
- ii) calculating the threshold value as the location of a peak of the second order curve.

9. A method for determining an unknown starting quantity of a target nucleic acid sequence in a test sample, the method comprising the steps of:

- 5 a) amplifying the target nucleic acid sequence and a first internal control in a first nucleic acid amplification reaction, wherein the first internal control comprises a second nucleic acid sequence different than the target nucleic acid sequence in the test sample;
- 10 b) amplifying a first standard and a second internal control in a second nucleic acid amplification reaction, wherein the first standard comprises a first known starting quantity of a calibration nucleic acid sequence different than the second nucleic acid sequence, and wherein the second internal control comprises the second nucleic acid sequence;
- 15 c) amplifying a second standard and a third internal control in a third nucleic acid amplification reaction, wherein the second standard comprises a second known starting quantity of the calibration nucleic acid sequence, the third internal control comprises the second nucleic acid sequence, and the starting quantity of the second nucleic acid sequence is substantially equal in each of the amplification reactions;
- 20 d) determining a respective threshold value for each of the standards, each of the internal controls, and the target nucleic acid sequence in the test sample;
- 25 e) normalizing the threshold value determined for the target nucleic acid sequence in the test sample to
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the threshold value determined for the first internal control;

5 f) normalizing the threshold values determined for the first and second standards to the threshold values determined for the second and third internal controls, respectively;

g) deriving a calibration curve from the known starting quantities and the normalized threshold values of the first and second standards; and

10 h) determining the starting quantity of the target nucleic acid sequence in the test sample using the calibration curve and the normalized threshold value determined for the target sequence.

15 10. The method of claim 9, wherein each of the threshold values comprises a cycle number.

11. The method of claim 9, wherein each of the threshold values comprises an elapsed time of amplification.

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12. The method of claim 9, wherein a respective threshold value is determined for each nucleic acid sequence by:

25 i) measuring, at a plurality of different times during amplification of the nucleic acid sequence, at least one signal whose intensity is related to the quantity of the nucleic acid sequence being amplified;

- ii) deriving a growth curve from the measurements of the signal;
- iii) calculating a derivative of the growth curve;
- iv) identifying a characteristic of the derivative; and
- 5 v) determining the threshold value associated with the characteristic of the derivative.

10 13. The method of claim 12, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a positive peak of the second derivative.

15 14. The method of claim 12, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a negative peak of the second derivative.

20 15. The method of claim 12, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a zero crossing of the second derivative.

25 16. The method of claim 12, wherein the step of calculating a derivative of the growth curve comprises calculating a

first derivative of the growth curve, and wherein the characteristic comprises a positive peak of the first derivative.

5 17. The method of claim 12, wherein the step of calculating  
a derivative of the growth curve comprises calculating  
second derivative values of the growth curve at a number  
of different measurement points in the reaction to yield  
a plurality of second derivative data points, the  
10 characteristic comprises a positive peak of the second  
derivative, and the step of determining the threshold  
value associated with the positive peak comprises:

- i) fitting a second order curve to the second  
derivative data points; and
- 15 ii) calculating the threshold value as the location of  
a peak of the second order curve.

18. The method of claim 9, wherein the step of normalizing  
the threshold value determined for the target nucleic  
20 acid sequence in the test sample to the threshold value  
determined for the first internal control comprises  
dividing the threshold value determined for the target  
nucleic acid sequence by the threshold value determined  
for the first internal control.

25 19. The method of claim 9, wherein the step of normalizing  
the threshold values determined for the first and second  
standards to the threshold values determined for the

second and third internal controls, respectively,  
comprises dividing the threshold values determined for  
the first and second standards by the threshold values  
determined for the second and third internal controls,  
5 respectively.

20. A method for determining an unknown starting quantity of  
a first nucleic acid sequence in a test sample, the  
method comprising the steps of:

- 10 a) amplifying in one reaction vessel the first nucleic  
acid sequence, a first standard, and a second  
standard, wherein the first standard comprises a  
known starting quantity of a second nucleic acid  
sequence different than the first nucleic acid  
15 sequence, and wherein the second standard comprises  
a known starting quantity of a third nucleic acid  
sequence different than the first and second  
sequences;
- 20 b) determining a respective threshold value for the  
first standard, the second standard, and the first  
nucleic acid sequence;
- c) deriving a calibration curve from the known starting  
quantities and from the threshold values determined  
for the first and second standards; and
- 25 d) determining the starting quantity of the first  
nucleic acid sequence using the calibration curve  
and the threshold value determined for the first  
nucleic acid sequence.



21. The method of claim 20, wherein each of the threshold values comprises a cycle number.

22. The method of claim 20, wherein each of the threshold values comprises an elapsed time of amplification.

23. The method of claim 20, wherein a respective threshold value is determined for each nucleic acid sequence by:

- i) measuring, at a plurality of different times during amplification of the nucleic acid sequence, at least one signal whose intensity is related to the quantity of the nucleic acid sequence in the vessel;
- ii) deriving a growth curve from the measurements of the signal;
- iii) calculating a derivative of the growth curve;
- iv) identifying a characteristic of the derivative; and
- v) determining the threshold value associated with the characteristic of the derivative.

24. The method of claim 23, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a positive peak of the second derivative.

25. The method of claim 23, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a negative peak of the second derivative.

26. The method of claim 23, wherein the step of calculating a derivative of the growth curve comprises calculating a second derivative of the growth curve, and wherein the characteristic comprises a zero crossing of the second derivative.

27. The method of claim 23, wherein the step of calculating a derivative of the growth curve comprises calculating a first derivative of the growth curve, and wherein the characteristic comprises a positive peak of the first derivative.

28. The method of claim 23, wherein the step of calculating a derivative of the growth curve comprises calculating second derivative values of the growth curve at a number of different measurement points in the reaction to yield a plurality of second derivative data points, the characteristic comprises a positive peak of the second derivative, and the step of determining the threshold value associated with the positive peak comprises:

- i) fitting a second order curve to the second derivative data points; and

- ii) calculating the threshold value as the location of a peak of the second order curve.

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